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Genetic diversity and phenotypic associations of feline caliciviruses from cats in Switzerland

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The genetic diversity and phenotypic associations of feline caliciviruses from cats in Switzerland --Manuscript Draft--

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The genetic diversity and phenotypic associations of feline caliciviruses from cats in Switzerland

Running title: Genetic diversity and phenotypic associations of FCV

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ABSTRACT

Feline calicivirus (FCV) is a common viral pathogen in domestic cats worldwide. The variable regions of the capsid (VP1) gene of FCV have one of the highest recorded rates of molecular evolution. Understanding the genetic diversity and phylogeny of FCV is a prerequisite to exploring the epidemiology and pathogenesis of this virus and to the development of efficacious vaccine strategies. In this study, we undertook a nationwide molecular characterization of FCV using for the first time nearly complete capsid (VP1) gene sequences. Sequences from 66 FCV samples were used to investigate the correlation between viral phylogeny and several traits, including geographic origin, signalment, husbandry, FCV vaccination, and co-infections. Codon-based nucleotide alignment showed that individual nucleotides and their corresponding amino acid sites were either invariant or highly variable. Using a threshold of 20% genetic distance in variable region E, FCV samples were grouped into 52 strains, 10 of which comprised 2 to 3 samples. Significant associations between FCV phylogeny and host characteristics were found, specifically the pedigree status of the cats, and two well-supported lineages were identified in which the current FCV strain definition was confounded. No correlation between viral genetic distances and geographic distances was evident. The greater resolution of the FCV phylogeny in this study compared to previous studies can be attributed to our use of more conserved regions of the capsid (VP1) gene; nonetheless, our results were still hampered by sequence saturation. The study highlights the need for whole genome sequences for FCV phylogeny studies.

Key words: Feline calicivirus, pedigree cat, phylogenetic analyses, trait correlation, capsid (VP1) gene, hypervariable region E

INTRODUCTION

Feline calicivirus (FCV) is a single-stranded, non-enveloped RNA virus belonging to the genus *Vesivirus* within the family *Caliciviridae* (Seal *et al.*, 1993). Three other genera are assigned to the *Caliciviridae*, namely the genera *Sapovirus*, *Lagovirus* and *Norovirus*. Some of these genera contain highly virulent species, for example human norovirus, which is a major cause of gastroenteritis (Robilotti *et al.*, 2015). FCV is a common viral pathogens of cats worldwide, and infections are associated with mucosal and cutaneous ulcerations, chronic oral inflammatory disease (gingivitis/stomatitis), pneumonia and a limping syndrome (Radford *et al.*, 2007). Outbreaks of highly virulent systemic FCV infections have been reported, in the USA (Foley *et al.*, 2006; Pedersen *et al.*, 2000; Schorr-Evans *et al.*, 2003) and subsequently in Europe (Battilani *et al.*, 2013; Meyer *et al.*, 2011; Reynolds *et al.*, 2009; Schulz *et al.*, 2011; Willi *et al.*, 2016). Affected cats develop a systemic inflammatory response syndrome and show high fever, subcutaneous oedema and skin ulcerations. Mortality rates of up to 60% have been reported for this syndrome (Pesavento *et al.*, 2004). Asymptomatic FCV infections have also been described, and up to 21% of healthy cats have been reported to test reverse-transcriptase (RT) PCR-positive for FCV (Berger *et al.*, 2015; Binns *et al.*, 2000; Fernandez *et al.*, 2016). Several studies have tried to identify FCV nucleotide or amino acid sequence variations that are unique to different disease manifestations, but the genetic basis of FCV pathogenicity remains unresolved (Coyne *et al.*, 2006b; Ossiboff *et al.*, 2007; Prikhodko *et al.*, 2014; Radford *et al.*, 2006; Rong *et al.*, 2006; Willi *et al.*, 2016).

The FCV genome comprises a 7.7 kb positive-sense RNA molecule that encodes three open reading frames (ORF). ORF 1 forms the viral replication complex, ORF 3 encodes the minor structural protein VP2 and ORF 2 encodes the major capsid protein VP1. ORF 2 can further be divided into six regions, A–F. Regions B, D and F are more conserved, whereas regions A, C and E are variable (Seal *et al.*, 1993).

Region E contains two hypervariable regions that are separated by a conserved domain (Seal *et al.*, 1993). Variable regions C and E of the capsid gene exhibit one of the highest evolutionary rates reported for RNA viruses (Coyne *et al.*, 2007b). These regions are of particular interest because they contain major neutralizing epitopes that define FCV antigenicity (Radford *et al.*, 1999; Tohya *et al.*, 1997). Previous studies showed that epidemiologically related FCV isolates differ at less than 20% of the nucleotide sites in the C and E regions (Radford *et al.*, 1997; Radford *et al.*, 2003; Radford *et al.*, 2000; Radford *et al.*, 2001). Based on this 20% genetic distance threshold for FCV strain definition, remarkably high strain diversity was identified in the cat population (Coyne *et al.*, 2012; Hou *et al.*, 2016). Phylogenetic analyses of variable regions C and E have been hampered by a lack of phylogenetic signal above the strain level caused by sequence saturation (Coyne *et al.*, 2012; Hou *et al.*, 2016). Previous studies were unable to draw reliable conclusions concerning phylogenetic relationships above the strain level or to correlate FCV phylogeny with clinical syndromes or the geographic location of infected cats (Coyne *et al.*, 2012; Geissler *et al.*, 1997; Glenn *et al.*, 1999).

To overcome this limitation, in this study, we undertook a nationwide molecular characterization of FCV samples using the nearly complete capsid (VP1) gene, which includes the more conserved regions B, D and F of the capsid (VP1) gene. We sequenced a total of 66 FCV samples from cats from 17 cantons of Switzerland that showed different clinical manifestations of FCV infection. We investigated the association between FCV gene sequences and the location, signalment, husbandry, clinical signs and vaccination history of the cats.

RESULTS

Characteristics of the study population

A total of 75 FCV samples collected during a previous nationwide FCV study (Berger *et al.*, 2015) and originating from four healthy cats and 71 cats with suspected infection were included in this study. Amplification of the PCR product and sequencing was successful for 66 FCV samples from three healthy cats and 63 cats with suspected infection (Tables 1-3). The 66 cats were sampled in 22 veterinary practices located in 17 out of 26 cantons in Switzerland (Fig. 1). The geographic distance (based on the postcodes) between the veterinary practices ranged from 4.7 km to 240 km (median = 92.4 km), and the distance between cat owners ranged from 0 km (same village) to 250 km (median = 95 km). Out of the 66 cats, 21 (32%) were pedigree cats; they belonged to seven different breeds (11 Maine Coons, 4 Norwegian Forest cats, 2 British Shorthair cats and 1 each of Oriental, Persian, Sacred Birman and Siamese cats). Fifty cats (78%) lived in multi-cat households, and 38 cats (59%) had outdoor access. Most of the cats were vaccinated against FCV ($n = 46$; 73%), and most had received at least a primary immunization (defined as two subsequent vaccinations within two to six weeks with the same vaccine strain, $n = 33$; 59%). Stomatitis, gingivitis or caudal stomatitis were the most common clinical signs ($n = 48$; 73%), and co-infections with other upper respiratory tract-associated pathogens were present in 44 (67%) of the cats.

FCV phylogeny and strain identification

Phylogenetic analysis included 66 nearly complete capsid (VP1) gene sequences (1700-1800 nucleotides (nt)) from cats, four FCV vaccine strains (FCV F9, FCV 255, G1 and 431) and 37 reference FCV sequences retrieved from GenBank. In general, the genetic heterogeneity among the capsid (VP1) gene sequences of the FCV samples was very high. The resulting codon-based nucleotide alignment showed that

individual nucleotides and their corresponding amino acid sites were either invariant or highly variable (data not shown). The estimated FCV phylogenies (Bayesian tree in Fig. 2; maximum likelihood (ML) tree in Fig. S1) were in accordance with these sequence properties. Specifically, there was a notable lack of phylogenetic structure, and most of the internal nodes close to the root were poorly supported, with Bayesian posterior probabilities of < 0.8 and ML bootstrap scores of $< 50\%$. A common property of these phylogenies was that their topologies exhibited short external branches and long internal branches. Further, the estimated Bayesian and ML topologies were incongruent. To quantify this, we placed the ML tree bootstrap scores onto the estimated Bayesian phylogeny (Fig. 2 and Fig. S1), which illustrates the topological incongruences between the two phylogenies, especially at more ancestral nodes. Nodes near the tree tips tended to be better supported, with high Bayesian posterior probabilities closer to 1.0 and ML tree bootstrap scores $> 50\%$.

Using a pairwise nucleotide genetic distance threshold of 20% in variable region E of the capsid (VP1) gene sequence, the samples were assigned to 52 strains. Most of the strains (81%) were defined by only one sample, with the exception of 10 strains (A-J) that contained two to three samples (Fig. 2). Within these strains containing multiple samples (A-J), the pairwise genetic distances in variable region E ranged from 2% to 20%. Remarkably, both the Bayesian and the ML phylogenies showed two well-supported lineages above the strain level (denoted lineages 1 and 2 in Fig. 2 and Fig. S1; Bayesian posterior probability = 1.0 and bootstrap scores $> 80\%$). Lineage 1 comprised samples from strains B and C and five singleton strains. Lineage 2 comprised samples from strains F-J plus fourteen singleton strains. Both lineages contained not only FCV samples from this study but also reference FCV sequences from a previous study from cats from Switzerland or Liechtenstein, which neighbours Switzerland (Willi *et al.*, 2016). Outside these two lineages, the nodes

that represent the common ancestor strains containing composite strains (i.e., strains A, D and E) were well-supported, with Bayesian posterior probabilities = 1.0 and ML bootstrap scores equal to 100%. Conversely, within lineages 1 and 2, the nodes that represent the common ancestor of each composite strain were well-supported in some instances (C, I and J) but poorly supported in others (B, F, G and H), indicating a difference between the genetic and patristic distances. The internal nodes within strains A, C, F and H tended to be supported with Bayesian posterior probabilities > 0.8 but not with bootstrap scores > 80%, whereas most of the nodes between the strains were poorly supported (Bayesian posterior probability < 0.8 and bootstrap scores < 50%).

The geographic origin of the FCV samples assigned to different strains and lineages is indicated in Fig. 3(a) and 3(b), respectively. The maximal geographic distance between FCV samples within a given strain (based on the postcodes of the cat owners) ranged from 0 km (strain J) to 209 km (strain F). Both samples of strain J (samples 32 and 145) were derived from one village, and all samples of strains A (samples 49, 83 and 106) and D (samples 68 and 155) were from the same canton in Switzerland.

All reference sequences retrieved from GenBank that were included in the phylogenetic analyses were placed outside strains A-J, except for the FCV vaccine strain F9, which was located in strain D together with two samples of this study (samples 68 and 155). These two samples were obtained from the same veterinary practice and from two cats that had received a FCV vaccination (with Feligen® CRP, Virbac, Glattbrugg, Switzerland; containing FCV F9 vaccine strain) 41 days and 70 days prior to sample collection.

Association of FCV phylogeny with different traits

All traits listed in Tables 1-3 and the "veterinary practice" trait were tested for association with FCV phylogeny. For each trait, we tested whether we could reject the null hypothesis that the trait is randomly distributed with respect to the tree topology.

A strong statistical association was found between the FCV phylogeny and the "pedigree" trait (Table 1; $p < 0.01$ using the association index (AI) and parsimony score (PS) statistics). Ancestral character state estimation of this trait showed that most of the FCV samples (17 out of 21) from pedigree cats belonged to lineage 2 and formed a monophyletic clade dominated by pedigree cats (Fig. 4). Within lineage 2, 10 of the 17 FCV samples from pedigree cats were derived from Maine Coon cats, which was the predominant breed in our study population. Within lineage 2, eight samples from pedigree cats belonged to strains F, G, H and I, and nine samples represented singleton strains. The remaining four samples from pedigree cats were singleton strains located outside of lineages 1 and 2. The association of FCV phylogeny with "pedigree cat" could not be explained by a geographical clustering of the pedigree cats because the cats originated from 11 different cantons, and the samples were collected in 14 different veterinary practices (Fig. 1). Furthermore, when the genetic distance of the samples from pedigree and non-pedigree cats was separately compared to geographic distances between owners or veterinary practices, no correlation was found (Fig. S2). We further assessed whether the pedigree cats in this study exhibited some characteristics that were different from the non-pedigree cats that could potentially account for the observed association of the "pedigree" trait with FCV phylogeny (Table S1). The analysis revealed that the pedigree cats were more commonly kept by cat breeders ($p_{\chi^2} < 0.01$), lived more

commonly in multi-cat households ($p_{\chi^2} = 0.02$) and without outdoor access ($p_{\chi^2} < 0.01$), and were less commonly treated with immunosuppressive ($p_{\chi^2} = 0.02$) and antibiotic medications ($p_{\chi^2} = 0.04$) than non-pedigree cats. The pedigree cats had more commonly received a primary immunization against FCV ($p_{\chi^2} = 0.03$), and the FCV vaccine strains used for immunization were different from those used for the non-pedigree cats ($p_{\chi^2} < 0.01$). Furthermore, pedigree cats more commonly suffered from lingual and oral ulcerations ($p_{\chi^2} < 0.01$; data not shown) than the non-pedigree cats.

There was also a significant association of the "veterinary practice" trait with FCV phylogeny ($p < 0.01$ using AI statistic, $p = 0.05$ using PS statistics). Ancestral state reconstruction indicated that some composite strains (strains A, D and J) were localised to individual veterinary practices; for strain C, two out of the three samples came from the same practice (Fig. 4). The time span between collections of closely related samples within one veterinary practice ranged from six days (strain C; samples 65 and 81) to seven weeks (strain J; samples 32 and 145). The FCV samples within the remaining six composite strains derived from different Swiss cantons and were collected in different veterinary practices. Overall, the strain diversity collected in each veterinary practice was high: in 19 out of 22 veterinary practices, the number of collected samples was equal to the number of FCV strains.

Looking at the different forms of husbandry type (private, cat breeder and others), the association with FCV phylogeny was inconclusive ($p = 0.02$ using the AI statistic; $p = 0.2$ using the PS statistic; Table 1). A questionable statistical association with FCV phylogeny was also found for the "primary FCV immunization" trait ($p = 0.06$ using the AI statistic, $p = 0.02$ using the PS statistic, Table 2). Finally, there was an inconclusive association of FCV phylogeny with the vaccine strain used for

immunization ($p = 0.04$ using the AI statistic, $p = 0.08$ using the PS statistic, Table 2). Because "pedigree" was significantly associated with husbandry type, primary immunization of the cats and the vaccine strain used for immunization (Table S1), these associations could have acted as confounding variables in the single-trait analyses. All other variables investigated in this study showed no significant association with FCV phylogeny (Tables 1 - 3).

Correlation between pairwise genetic and geographical distances

Given the weak statistical support for many phylogenetic nodes, we further explored the spatial clustering of FCV samples by plotting pairwise genetic distances against pairwise geographical distances (Fig. 5). Pairwise geographical distances were calculated between the locations of the cat owners and also between the locations of the veterinary practices where the samples had been collected. We observed weak associations between genetic and spatial distances in both cases (Pearson correlation $r = 0.036$, $p = 0.245$ for distances between cat owners; Pearson correlation $r = 0.033$, $p = 0.268$ for distances between veterinary practices).

DISCUSSION

Investigating the genetic diversity and phylogeny of FCV is a prerequisite to understanding the epidemiology and pathogenesis of FCV and may assist in the development of efficacious vaccine strategies. This study provides, for the first time, a nationwide analysis of FCV samples based on the nearly complete capsid (VP1) gene. We identified 52 different strains circulating in the Swiss cat population and two well-supported lineages above the strain level that contained 24 and 9 samples. The high strain diversity observed in the present study is in agreement with three recent studies that addressed FCV phylogeny within communities (Coyne *et al.*, 2007a), at the country level in the UK (Coyne *et al.*, 2012) and in different European countries

(Hou *et al.*, 2016). These three studies were all based on partial capsid (VP1) gene sequences (i.e., variable regions C and E of the ORF2 of FCV) and were hampered by a lack of phylogenetic signal above the strain level caused by sequence saturation. To overcome this limitation, in this study, we analysed the almost complete capsid (VP1) gene, which included more conserved domains of the capsid protein. The resolution of the phylogeny in our study was thus greater than in previous studies, but there was still a lack of deep phylogenetic structure; most internal nodes close to the phylogeny root were poorly supported, reflecting the exceptionally polymorphic nature of variable sites within the FCV capsid (VP1) gene.

In accordance with previous studies, a FCV strain was defined by > 20% genetic distance in variable region E of the capsid (VP1) gene and included 235 bp of variable region E. This definition is based on studies that showed that related FCV samples in endemically infected colonies show up to 16% genetic distance, whereas unrelated samples show > 20% distance in variable region E of VP1 (Radford *et al.*, 1997; Radford *et al.*, 2003). The FCV strain definition therefore applies to closely related samples that are epidemiologically and spatially linked. However, it is sometimes used ambiguously, as previous studies applied the genetic distance definition to 235 bp, 420 bp or 529 bp amplicons of variable regions C and E (Coyne *et al.*, 2006a; Coyne *et al.*, 2007b; Hou *et al.*, 2016; Radford *et al.*, 1997; Radford *et al.*, 2000). These differences are important given the very high variation in genetic diversity among sequence regions within the capsid (VP1) gene. Our analyses also identified two well-supported lineages above the strain level, lineages 1 and 2, that included 10 and 26 FCV samples, respectively. The samples within lineages 1 and 2 showed 15 - 33% and 12 - 34% pairwise genetic distances, respectively, in variable region E. Sequences with genetic distances >20% that cluster together with

bootstrap scores >80% have so far only been documented in one study of cats in the UK (Coyne *et al.*, 2012).

The present study documents for the first time a lack of FCV genetic divergence, e.g. within the pedigree trait. There was a strong statistical association between FCV phylogeny and the “pedigree” trait and ancestral character state estimation revealed that 17 out of 21 FCV samples from pedigree cats belonged to lineage 2 and formed a monophyletic and well-supported clade mostly comprising pedigree cats, indicating an epidemiological link between these samples. It could be hypothesized that pedigree cats represent a new environmental niche to which FCV might become adapted. The association with pedigree might be caused by a common route of transmission of FCV strains among pedigree cats in Switzerland, e.g., during cat exhibitions or within breeding catteries. FCV is a highly contagious pathogen and resistant to many disinfectants. An indirect transmission (without cat-to-cat contact) at cat exhibitions could take place if hygienic measures are suboptimal. Alternatively, kittens could have acquired the infection within a few breeding catteries and still carry the virus as adult cats since FCV can induce persistent, long-term infections (Coyne *et al.*, 2006a; Wardley & Povey, 1977). In line with this, most pedigree cats in this study were Maine Coon cats, and 10 out of 11 FCV samples from Maine Coon cats were located in lineage 2. There is no information whether the Maine Coon cats of this study trace back to a few breeding catteries. However, the Swiss Maine Coon association lists 24 official breeders (Maine Coon Association, 2016). It seems improbable that all Maine Coon cats of this study originated from very few breeding catteries. Furthermore, FCV samples from other cat breeds in this study were also predominantly placed in lineage 2. Another explanation for the limited genetic diversity of FCV within pedigree cats could be that some host traits, e.g. the genetic background of pedigree cats, might increase the susceptibility to certain virus strains

and/or limit replication of other strains. This has been shown for other feline pathogens. Cheetahs, which experienced a severe population bottleneck in their evolutionary history, have a higher vulnerability to feline infectious peritonitis caused by feline coronavirus (O'Brien *et al.*, 1985). As another example, the viral replication of exogenous FeLV has been shown to be related to endogenous FeLV loads in domestic cats (Tandon *et al.*, 2008).

Viral quasispecies formation has been documented for FCV (Radford *et al.*, 1998), and co-infections with two different FCV strains within individual cats have occasionally been reported (Coyne *et al.*, 2006c). PCR amplification and direct sequencing, as applied in this study, is limited in its ability to detect quasispecies formation and viral variants that are present at only low levels within a host. Newer sequencing techniques, i.e. high-throughput sequencing, are able to detect minority variants that are present in only 1-2% of sequence reads (Radford *et al.*, 2012), but these methods have not yet been applied to FCV.

In agreement with a previous study (Coyne *et al.*, 2012), we found only weak associations between the genetic and spatial distances of FCV samples. This was the case for both the location of the cat owners and the location of the veterinary practices where the samples had been collected. FCV strains have been shown to be confined to close geographic regions (Coyne *et al.*, 2012; Hou *et al.*, 2016). Coyne *et al.* found only two FCV strains detected >100 km apart, and the most widespread strain contained only variants of FCV F9, a common FCV vaccine strain (Coyne *et al.*, 2012). These results suggest that FCV has only limited ability for widespread geographic spread. In our study, all but one composite strain and both lineages 1 and 2, contained only samples from cats from Switzerland and Liechtenstein, a neighbouring country of Switzerland. However, the maximal geographical distance

between FCV samples of composite strains was 209 km, and geographic distances of >100 km within a composite strain were not uncommon. Furthermore, we only identified two FCV F9 variants, and they were not geographically dispersed but were collected from two cats in the same veterinary practice. There could be several reasons for the differences between our results and those of Coyne *et al.* (Coyne *et al.*, 2012). First, the shorter distances between sampling sites in our study (median 92.4 km) compared to the UK study (over 300 km for the majority of practices) allowed us to also explore intermediate distances of FCV dissemination. Second, our study included a higher percentage (0.004%) of the total national cat population (estimated 1.5 million cats, Schweizer Tierschutz, 2005) than the UK study (0.002%, estimated 8 million cats, Pet food manufacturer's association, 2012), which might have allowed us to detect less prevalent FCV strains. Third, only one FCV sample per household was included in our study, whereas samples collected from cats from the same household were included in the UK study, which could have altered the geographical footprint of FCV. Fourth, our study excluded cats with recent FCV vaccination to avoid the inclusion of FCV vaccine strains that can occasionally be detected up to three weeks after vaccination (Bennett *et al.*, 1989; Pedersen & Hawkins, 1995; Ruch-Gallie *et al.*, 2011). The most geographically widespread FCV strains in our study (strains F to I) were predominantly obtained from pedigree cats. Pedigree cats might be moved over longer geographic distances, i.e., for breeding or for cat exhibitions. The inclusion of a relatively large number of pedigree cats in our study could therefore also account for the observed wide geographic dispersal of some FCV strains.

Although the overall strain diversity collected in veterinary practices was high, we found a significant association between the “veterinary practice” trait and the FCV phylogeny. Several composite strains (A, C, D and J) were almost exclusively

collected in single veterinary practices. The existence of closely related FCV samples in one veterinary practice has also been previously reported (Coyne *et al.*, 2012). FCV is highly resistant to many disinfectants (Radford *et al.*, 2009), and if hygiene measures are insufficient, indirect transmission of FCV could play a role in the practice environment. However, this would require that the cats were presented and infected in the veterinary practices prior to sample collection. Alternatively, contamination of swabs with FCV from the practice environment could have caused RT-PCR-positive results. However, all except one of the cats from which these closely related samples were obtained showed clinical signs consistent with FCV infection. Furthermore, all sample collection material was provided to the veterinary practices together with detailed instructions on how to properly collect the samples. Finally, the detection of closely related FCV samples in one veterinary practice could reflect the transmission of this FCV strain between cats in the catchment area of that practice. The cats infected with variants of the composite strains A, D or J lived in rather close proximity to each other (within 16 km apart), although this was not the case for the cats infected with variants of strain C (up to 62 km apart). Furthermore, only five out of nine cats in strains A, C, D and J were allowed outdoors. However, FCV can also be indirectly transmitted between cats via fomites, which could have accounted for the infections of the indoor cats.

Only two samples closely related to the FCV vaccine strain F9 were detected in this study (samples 68 and 155, strain D); the sequences showed <3% genetic divergence from the vaccine strain in the variable region E. Both samples were collected in a single veterinary practice and derived from two cats that had been vaccinated with a vaccine containing FCV F9 41 and 70 days prior to sample collection, respectively. FCV F9 is contained in several modified-live virus vaccines licensed in Switzerland, and a short-term oral shedding of FCV F9 for some days up

377 to three weeks after vaccination has been reported (Bennett *et al.*, 1989; Pedersen &
378 Hawkins, 1995; Ruch-Gallie *et al.*, 2011). Previous studies have also occasionally
379 reported the isolation of FCV F9 variants from the general cat population (Abd-
380 Eldaim *et al.*, 2005; Coyne *et al.*, 2012; Coyne *et al.*, 2006a), but in contrast to the
381 present study, recent vaccination was not an exclusion criterion in these studies. The
382 detection of FCV F9 variants in the present study up to 70 days after vaccination
383 could be explained by an inadvertent infection of the cats with the FCV vaccine
384 strain, as reported after the licking of accidentally spilled vaccine material from the fur
385 of the cats. In such cases, clinical signs of FCV infection and prolonged shedding of
386 the vaccine virus (several weeks to a few months) can occur (Pedersen & Hawkins,
387 1995). Alternatively, the two cats might have been exposed to an FCV F9-like field
388 variant, but this seems less likely because cat 155 was kept strictly indoors as a
389 single cat and lived 16 km away from cat 68. Finally, prolonged shedding of FCV F9
390 after the correct application of the vaccine might occur in cats with a severe immune
391 deficiency. While cat 68 was a healthy young cat presented for castration, cat 155
392 presented with fever, apathy, ocular discharge and gingivitis and tested positive for
393 feline immunodeficiency virus in RT-PCR (Berger *et al.*, 2015). However, it seems
394 improbable that immunosuppression due to FIV infection explains the extended time
395 of shedding of FCV after vaccination in cat 155. Prolonged FCV shedding was not
396 observed in FIV-positive cats in an experimental study (Reubel *et al.*, 1994), and
397 severe immunodeficiency usually occurs only during long-term FIV infection
398 (Hofmann-Lehmann *et al.*, 1997); cat 155 was only five months old at the time of
399 sampling. Overall, the detection of modified-live FCV vaccine strains in field cats was
400 a rare finding and the clinical significance can be assumed to be small. However,
401 vaccination with certain vaccine strains was suspected to drive the emergence of
402 strains not covered by vaccine-induced immune protection (Ohe *et al.*, 2007).

Because FCV antigenetic characteristics do not necessarily correlate with capsid VP1 gene sequences (Geissler *et al.*, 1997; Poulet *et al.*, 2000), the role of immune-escape in the emergence of novel strains could not be evaluated in the present study. However, none of the commercial vaccine strains included in the phylogenetic analyses were phylogenetic outliers and all vaccine strains remained within the genetic diversity of the samples sequenced in this study.

CONCLUSION

Our study indicates that FCV lineages above the strain level can sometimes be identified if genetic data from more conserved regions of the FCV genome are included in phylogenetic analyses. FCV phylogeny was significantly associated with the pedigree status of the sampled animals, and the samples from most pedigree cats were placed in lineage 2 in this study. All but one strains and both lineages identified in this study were restricted to cats in Switzerland or Liechtenstein. Within Switzerland, we observed a greater geographic dispersal of FCV strains than previously reported. Variants of the FCV F9 vaccine strain were very rarely detected and may be assigned to the inadvertent infection of cats by oral intake of spilled vaccine material. The resolution of the FCV phylogeny in this study was greater than that in previous studies, which can be attributed to the use of more conserved regions of the capsid (VP1) gene. Nonetheless, phylogenetic analyses were still hampered by sequence saturation. To overcome this limitation and to further resolve the phylogenetic relationships of FCV, future studies should extend genetic analyses to longer sequences of FCV.

MATERIALS AND METHODS

Study setup, sample collection and processing

The samples available for this study were collected during a nationwide FCV study in Switzerland (Berger *et al.*, 2015). For this purpose, oropharyngeal cytobrushes, and nasal and conjunctival swabs were collected from 200 cats with suspected FCV infection and from 100 healthy cats (Berger *et al.*, 2015). The sample size included in the original study was determined using Epi Info™ v.7 (Centers for Disease Control and Prevention, 2016) on the basis of an estimated Swiss cat population of 1.5 million (Schweizer Tierschutz, 2005) and 26 % infection prevalence (Binns *et al.*, 2000). Only one cat per owner was included. Cats that had been vaccinated within 21 days prior to collection were excluded. Samples from each cat were pooled, and total nucleic acid (TNA) was extracted (Berger *et al.*, 2015). The data available for each cat contained information on the geographic origin, demographic data (age, sex, reproductive status, pedigree and breed), husbandry data (type of husbandry, such as private, cat breeder and others, group housing, outdoor access), vaccination history, medical treatments and clinical signs (Berger *et al.*, 2015). Two suspected FCV cases (samples 65 and 185) showed clinical signs resembling virulent systemic disease; genetic data from these cats were obtained during a previous study (Willi *et al.*, 2016). To compare our genetic data with that of FCV vaccine strains, RNA was extracted from two FCV vaccines (Feligen® CRP, Virbac; Nobivac® Tricat III, MSD Animal Health GmbH, Luzern, Switzerland) using a QIAamp® Viral RNA kit (Qiagen, Hombrechtikon, Switzerland).

PCR assays

Within the previous study (Berger *et al.*, 2015), TNA was tested for the presence of FCV with two real-time RT-PCR assays (Abd-Eldaim *et al.*, 2009; Helps *et al.*, 2002),

named S1 and S2, that were recently optimized (Berger *et al.*, 2015). All cats that tested positive in at least one of the two RT-PCR runs were categorized as FCV-positive. Moreover, for each sample, the real-time PCR results for the detection of FHV-1, *Chlamydia felis*, *Mycoplasma felis* and *Bordetella bronchiseptica* were available (Berger *et al.*, 2015).

Synthesis of cDNA, conventional PCR amplification and sequencing

FCV-positive samples with high viral loads (cycle threshold values < 30.0; n = 75) in RT-PCR S1 or S2 and two commercial FCV vaccines (see above) were used for sequencing. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) under the following conditions: 10 min 25°C, 120 min 37°C, 10 min 70°C. The reaction mixture consisted of 2.5 µl 10× RT Buffer, 2.5 µl 10× RT random primers, 1.0 µl 25× dNTP Mix (100 mM), 1.25 µl MultiScribe® Reverse Transcriptase (50 U/µl), 0.3125 µl RNasin® Plus RNase Inhibitor (40 U/µl) (Promega AG, Dübendorf, Switzerland), and 10 µl template TNA, brought up to 25 µl with RNase/DNase-free molecular biology grade water (Axon Lab AG). FCV cDNA was amplified with Phusion™ Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, USA) using the published primers AoS (forward) and AoA (reverse) (Ohe *et al.*, 2006), which results in the amplification of 1945 nt of the FCV capsid (VP1) gene (equivalent to nucleotide positions 5326 to 7270 of the FCV F9 reference strain; GenBank accession number M86379). The PCR reaction comprised 5 µl 5× Phusion HF Buffer, 0.5 µl dNTPs (10 mM), 0.625 µl primer AoS (20 µM) and 0.625 µl primer AoA (20 µM), 0.25 µl Phusion Hot Start II Polymerase (2 U/µl) and 2.5 µl of template cDNA brought up to 25 µl. The PCR thermal cycling conditions were as follows: 30 sec at 98°C, 40 cycles of 10 sec at 98°C, 30 sec at 53°C and 90 sec at 72°C, followed by 10 min at 72°C.

475 In 17 samples, PCR amplification with the above-mentioned protocol failed. These
476 samples were therefore amplified with the SuperScript® III One-Step RT-PCR
477 System and the Platinum® *Taq* DNA polymerase (Invitrogen, Basel, Switzerland)
478 using the primers AoS and AoA (Ohe *et al.*, 2006), which resulted in the successful
479 amplification of 8/17 samples. The PCR reaction mixture contained: 12.5 µL 2×
480 reaction Mix, 0.625 µL RNasin® Plus RNase Inhibitor (40 U/µL), 0.25 µL primer AoS
481 (20 µM) and 0.25 µL primer AoA (20 µM), 1.0 µL SuperScript® III Platinum® *Taq*
482 DNA polymerase and 5 µL of template TNA made up to 25 µL. The thermal cycling
483 conditions were as follows: 60°C for 30 min and 94°C for 2 min, followed by 40 cycles
484 of 94°C for 15 sec, 53°C for 30 sec and 68°C for 150 sec, and a final elongation by
485 68°C for 5 min. The synthesis of cDNA and the conventional PCR were run on a
486 Biometra TPersonal thermal cycler (BioLabo Scientific Instruments, Châtel-Saint-
487 Denis, Switzerland).

488 The PCR products were separated on a 1.5% agarose gel, and bands of the
489 appropriate size (1945 bp) were excised and eluted with a QIAquick® Gel extraction
490 kit (Qiagen). For sequencing (Microsynth, Balgach, Switzerland), the amplification
491 primers AoS and AoA (Ohe *et al.*, 2006) and newly designed internal primers were
492 used (S_FCV_La.543f: 5'-GCT-TGG-TCT-GGM-TCT-ATT-GA-3'; S_FCV_Fl.1265r:
493 5'-GCC-AAC-CAT-CAG-GTA-TGC-CT-3'; FCVSeq_6145_6164f: 5'-CAY-YTD-ATG-
494 TCT-GAY-ACT-GA-3'; FCVSeq_5749_5768f: 5'-GAR-CCH-ARY-KCH-CAA-ATG-TC-
495 3'; FCVSeq_6705_6725r: 5'-GGR-ATK-GTD-GTR-TCD-GGC-CA-3'). Because direct
496 sequencing of the capsid (VP1) gene was not successful in five samples (Nos. 52,
497 68, 100, 145 and 155), the PCR products of these samples were cloned using a
498 Topo® TA Cloning® Kit with the pCR™ II-TOPO® vector and TOP10F' One Shot®
499 *E.coli* bacteria (Invitrogen, Basel, Switzerland). Correct insertion of the PCR product
500 was checked by EcoRI (Thermo Scientific) digestion analysis, and plasmid DNA was

purified with a Qiaprep® Spin Miniprep Kit (Qiagen). The inserted PCR products were sequenced (Microsynth).

Sequence alignment and phylogenetic analyses

All sequences were assembled, and a consensus sequence for each sample was obtained using Geneious 7.1.8 (Kearse *et al.*, 2012). The capsid (VP1) gene sequences of the vaccine strains FCV G1 and FCV 431 were provided by the manufacturer (Merial, Lyon, France). Reference FCV sequences from Europe, North America, Asia and Oceania were retrieved from GenBank. The European reference sequences included sequences from four cats from Switzerland or Liechtenstein that showed clinical signs compatible with virulent systemic disease (Willi *et al.*, 2016). Codon-based multiple alignment of the capsid (VP1) nucleotide sequences was performed using MAFFT (Kato & Standley, 2013). Nucleotide sequence saturation tests were performed in Dambe (Xia, 2013), which implements Xia *et al.*'s test of nucleotide substitution saturation (Xia & Lemey, 2009; Xia *et al.*, 2003). This test indicated that the 3rd codon positions in the nucleotide alignment were not saturated. The full nucleotide alignment was retained and used in phylogenetic analyses.

Bayesian and ML phylogenetic trees were estimated from the nucleotide alignment with MrBayes (Huelsenbeck & Ronquist, 2001) (with 10,000,000 generations and 25% burnin) and RAxML (Stamatakis, 2006), respectively. In both cases, the GTR + G + I substitution model and parameters selected by jModelTest2 were used (Darriba *et al.*, 2012). Statistical support for nodes in the ML phylogeny was assessed using a bootstrap approach with 100 replicates. The trees were midpoint rooted. Given the known sampling dates of each sample, the temporal signal of the phylogenies was assessed using TempEst (Rambaut *et al.*, 2016). A regression of the sampling date against the root-to-tip genetic distances indicates that the data set contains

insufficient temporal signal to justify the use of a phylogenetic molecular clock model (data not shown). The samples were classified into FCV “strains” using the previously defined nucleotide distance threshold of 20% for variable region E of the capsid (VP1) gene (Radford *et al.*, 1997; Radford *et al.*, 2000). Pairwise genetic distances among sequences were calculated using the function “dist.dna” (Jukes-Cantor model, JC69) implemented in the R package “ape” (Paradis *et al.*, 2004). Lineages were defined as well-supported clades containing at least two strains containing multiple samples.

Phylogeny-trait correlation and ancestral character estimations

Possible correlations between phylogenetic tree structure and trait values for each sample (e.g., sampling location, vaccination status; see Tables 1-4) were assessed using the methods implemented in BaTS (Parker *et al.*, 2008). Trait values were randomized 100 times to yield a null distribution for hypothesis testing. A correlation was considered unambiguously positive if both the AI and PS statistics rejected the null hypothesis with $p \leq 0.01$. Phylogenetic uncertainty was taken into account by using the set of tree topologies estimated by Bayesian phylogenetic inference (MrBayes). For those traits that were significantly clustered on the FCV phylogeny, parsimonious ancestral character state estimation was performed using the function “pace” implemented in the R package “phangorn” (Schliep, 2011).

Comparison between genetic and geographical distances

Pairwise genetic distances between sequences were calculated as described above. Geographical distances between each pair of samples were calculated using the postcodes for each sample and an online distance calculator (GlobeFeed, 2014). A quadratic assignment procedure correlation was used to test for a relationship between the genetic distance (%) and the geographical distance using the function

“qaptest” implemented in the R package “sna” (Butts, 2014). This method computes standard measures of correlation between the genetic and geographic distance matrices and then computes an estimate of the significance of the correlation by permuting the elements of one of the matrices 5,000 times and counting the number of correlations between the observed and permuted matrices that are larger or smaller than the empirical estimate. The genetic and geographic distance matrices were plotted and visualized using the function “hexbin” implemented in the R package “hexbin” (Carr, 2015).

Statistical analysis

The exposure variables between pedigree and non-pedigree cats were compared using a Chi-squared (p_{χ^2}) test or a Fisher’s exact test (p_F) for small numbers ($n < 5$) and Analyse-it® for Microsoft Excel 4.51 (Analyse-it Software Ltd., Leeds, United Kingdom). Proportions and 95% confidence intervals (CI) were calculated using GraphPad Prism® version 6 for Windows (GraphPad Software, San Diego, CA, USA). Variables such as the age of the cats were compared between the two groups using the Wilcoxon-Mann-Whitney test (p_{MWU}). P-values < 0.05 were considered statistically significant. The maps were produced using QGIS Geographic Information System (version 2.8.1)(QGIS Development Team, 2015). Canton boundaries were obtained from the Swiss Federal Office of Topography (Bundesamt für Landestopographie, 2015).

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FIGURE LEGENDS

Figure 1: Map of Switzerland and its cantons, showing the origin of the FCV samples sequenced in this study. Coordinates were calculated using the home address of the cat owners, and the map was produced using QGIS (QGIS Development Team, 2015).

Figure 2: Bayesian phylogenetic tree estimated from 109 FCV capsid (VP1) gene nucleotide sequences, including reference sequences and sequences generated in this study. Statistical support for phylogenetic nodes corresponds to the Bayesian posterior probabilities and ML bootstrap scores (100 replicates, see Fig. S1),

separated by the slash character. Hyphens replace posterior probabilities <0.8 and bootstrap scores $<50\%$. Tip labels are coloured by sampling locations: Swiss samples from this study are in red, European reference sequences in green, North American reference sequences in blue, Oceanian reference sequences in purple, and Asian reference sequences in brown. Grey boxed areas denote composite strains A to J (for details see text). Minimum-maximum genetic diversity (%) and minimum-maximum geographical distances (km) within each composite strain are indicated next to the corresponding strain name. Circled numbers indicate denoted lineages 1 and 2.

Figure 3: Maps of Switzerland and its cantons displaying (a) the distribution of the composite and singleton strains and (b) the distribution of the FCV samples assigned to lineage 1, 2 or no lineage. Both samples of strain J originated from the same village and are indicated as a single dot in Fig. 3(a). Coordinates were calculated using the home address of the cat owners, and the map was produced using QGIS (QGIS Development Team, 2015).

Figure 4: Bayesian FCV phylogenetic tree upon which ancestral trait state reconstructions have been superimposed. Tip circles in red and in blue show FCV isolated from non-pedigree cats and pedigree cats, respectively. Node circles indicate the parsimonious ancestral trait state reconstruction of the “pedigree” trait. Dashed branches represent lineages for which ancestral character state reconstruction of the “veterinary practice” trait was non-ambiguous. The colours of the dashed branches correspond to the veterinary practices.

Figure 5: Comparison of pairwise genetic distances (%) and pairwise geographical distances obtained from the samples in this study using variable region E of the capsid (VP1) gene. Panel A shows the correlation obtained if the pairwise geographic

distances are calculated between the locations of the cat owners, and panel B shows the correlation obtained if the pairwise geographic distances are calculated between the locations of the veterinary practices.

Figure S1: Maximum likelihood phylogenetic tree estimated from 109 FCV capsid gene nucleotide sequences, including reference sequences and sequences generated in this study. Statistical support for the phylogenetic nodes was assessed using a bootstrap approach (100 replicates).

Figure S2: Comparison of pairwise genetic distances (%) and pairwise geographical distances obtained from the samples in this study based on variable region E of VP1. Panels A and C show the correlations obtained if the pairwise geographic distances were calculated between the locations of the cat owners and included only samples isolated from non-pedigree cats. Panels B and D show the correlations obtained if the pairwise geographic distances were calculated between the locations of the veterinary practices and included only samples isolated from pedigree cats.

Tables

Table 1: Characteristics of the 66 FCV-positive cats and association with FCV

phylogeny. *P* values < 0.05 are shown in bold.

Parameter	Median	Lower 95% CI [#]	Upper 95% CI [#]	AI ^{&} (P value)	PS [§] (P value)
Age (years)	3.8	0.25	13.7		
Parameter	Proportion	Lower 95% CI [#]	Upper 95% CI [#]	AI ^{&} (P value)	PS [§] (P value)
Sex (male)	0.62	0.49	0.74	0.13	0.26
Intact reproductive status*	0.46	0.33	0.59	0.62	0.81
Pedigree	0.32	0.21	0.44	< 0.01	< 0.01
Breed				< 0.01	< 0.01
Maine Coon	0.17	0.09	0.28		
British Shorthair	0.03	0.00	0.11		
Norwegian Forest Cat	0.06	0.02	0.15		
Other breeds	0.06	0.02	0.15		
Multi-cat household*	0.78	0.66	0.88	0.56	0.24
Group size*				0.22	0.23
with 2-3 cats/group	0.40	0.28	0.53		
with 4-5 cats/group	0.16	0.08	0.27		
>5 cats/group	0.22	0.13	0.35		
Husbandry type				0.02	0.2
Private	0.79	0.67	0.88		
Cat breeder	0.09	0.03	0.19		
Other husbandry	0.12	0.05	0.23		
Outdoor access*	0.59	0.46	0.71	0.56	0.12
Immunosuppressive therapy	0.15	0.08	0.26	0.43	0.31
Antibiotic therapy	0.32	0.21	0.44	0.16	0.23
Antiviral therapy*	0.03	0.00	0.11	0.25	1.0

[#]CI, confidence interval, [&]AI, association index, [§] PS, parsimony score, *data missing for

single cats (reproductive status, n = 5; multi-cat household, n = 2; group size, n = 3; outdoor access, n = 1, antiviral therapy, n = 1)

Table 2: FCV vaccination status of the 66 FCV-positive cats and association with FCV phylogeny. *P* values < 0.05 are shown in bold.

Parameter	Proportion	Lower 95% CI [#]	Upper 95% CI [#]	AI ^{&} (P value)	PS [§] (P value)
FCV-vaccinated*	0.73	0.43	0.68	0.38	0.25
FCV vaccine strain*				0.04	0.08
Only F9	0.32	0.21	0.45		
Only G1/431	0.06	0.02	0.16		
Others	0.35	0.23	0.48		
Primary immunization [‡]	0.59	0.45	0.72	0.06	0.02

[#]CI, confidence interval, [&]AI, association index, [§]PS, parsimony score, *data missing for single cats (FCV vaccination status, n = 3; FCV vaccine strain, n = 3; primary immunization, n = 10), [‡]Primary immunization was defined as two subsequent vaccinations within 2 to 6 weeks with the same FCV vaccine strain

Table 3: Health status, clinical signs and co-infections with other upper respiratory tract-associated pathogens in the 66 FCV-positive cats and association with FCV phylogeny.

Parameter	Proportion	Lower 95% CI [#]	Upper 95% CI [#]	AI ^{&} (P value)	PS [§] (P value)
Healthy	0.05	0.01	0.13	0.31	1.0
Sneezing and/or nasal discharge*	0.32	0.21	0.45	0.61	0.93
Conjunctivitis and/or ocular discharge	0.39	0.28	0.52	0.44	0.31
Salivation	0.38	0.26	0.51	0.79	0.87
Lingual and/or oral ulcerations	0.30	0.20	0.43	0.79	0.31
Stomatitis and/or gingivitis and/or caudal stomatitis	0.73	0.60	0.83	0.25	0.52
Cutaneous ulcerations and/or cutaneous oedema	0.05	0.01	0.13	0.07	1.0
Limping and/or swollen joints	0.06	0.02	0.15	0.25	1.0
<i>M. felis</i> PCR-positive	0.56	0.43	0.69	0.06	0.06
FHV-1 PCR-positive	0.23	0.13	0.35	0.25	0.62
<i>C. felis</i> PCR-positive	0.05	0.01	0.13	0.25	1.0
<i>B. bronchiseptica</i> PCR-positive	0.05	0.01	0.13	0.43	1.0

[#]CI, confidence interval, [&]AI, association index, [§]PS, parsimony score, *data from one cat missing

Fig. 1

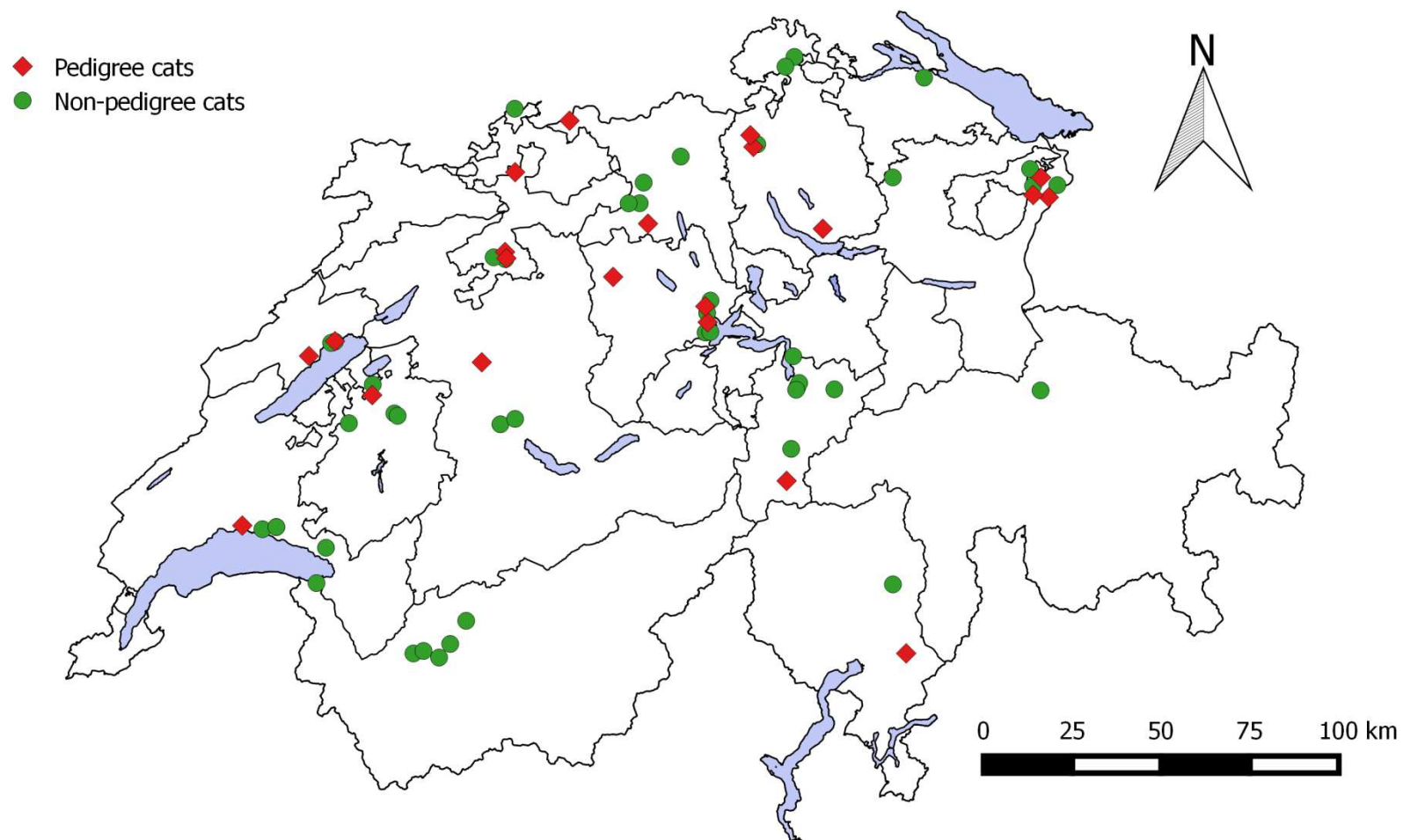


Fig. 2

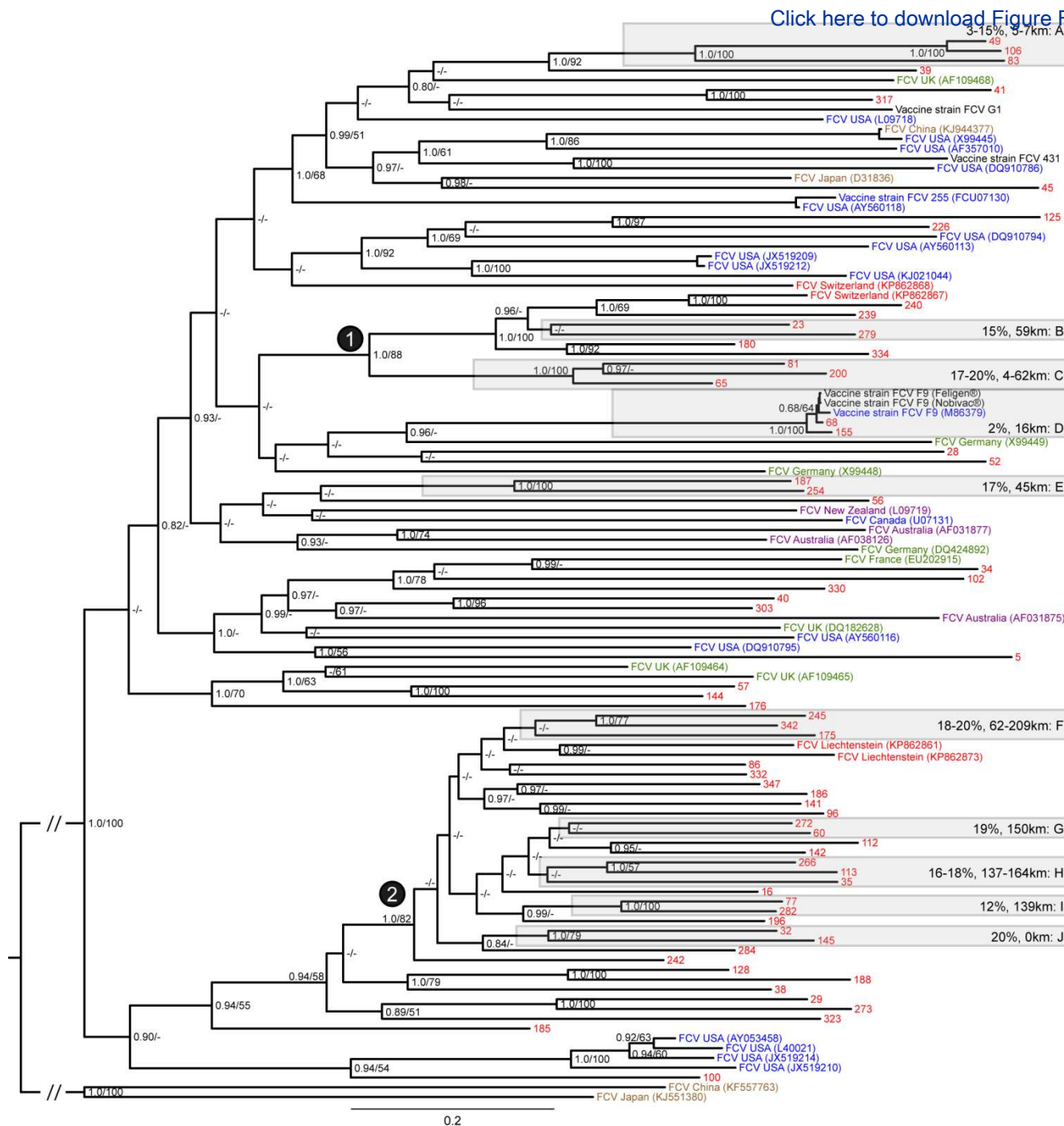


Fig. 3(a)

- strain A
- strain B
- strain C
- strain D
- strain E
- strain F
- strain G
- strain H
- strain I
- strain J
- singleton strains

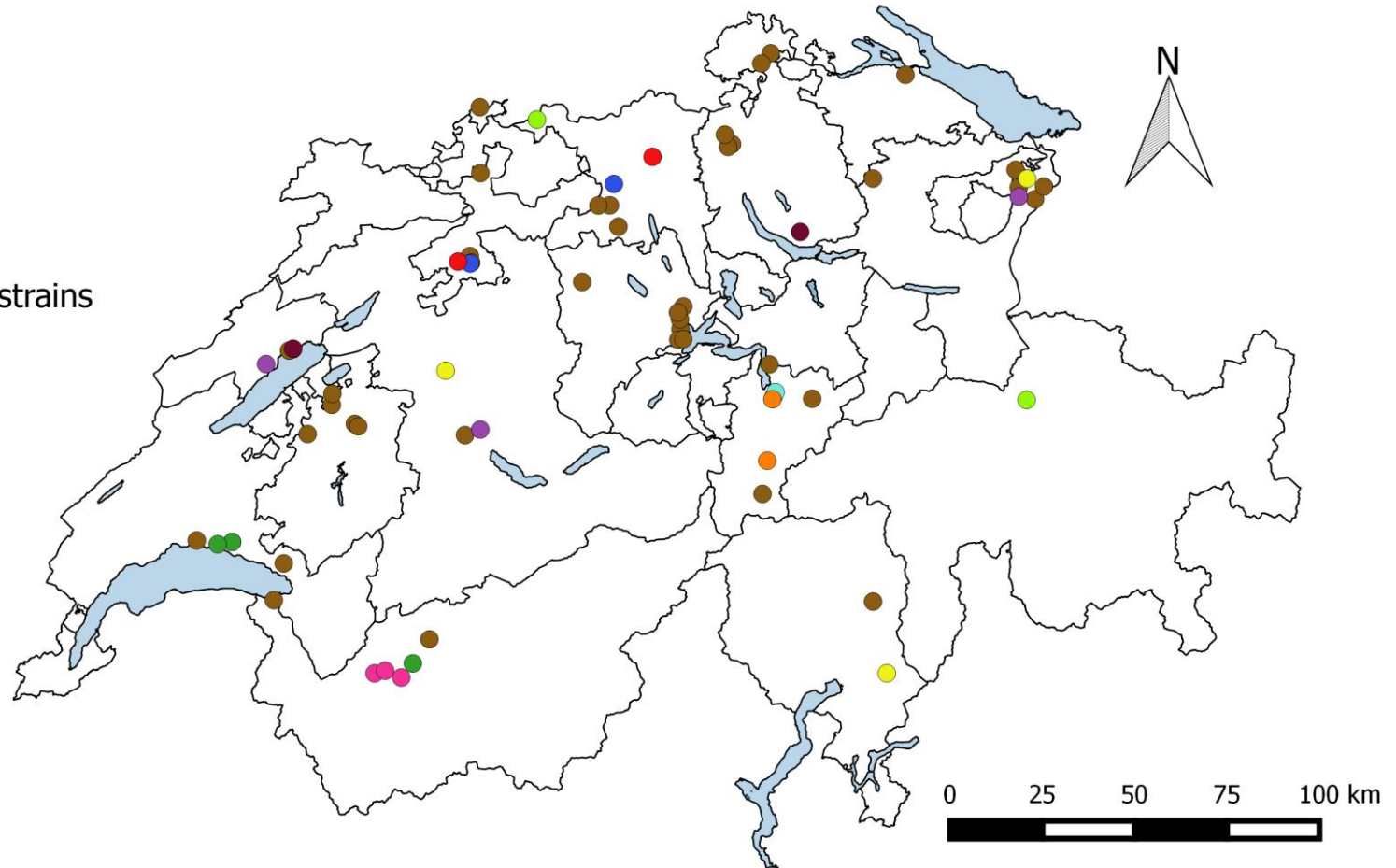


Fig. 3(b)

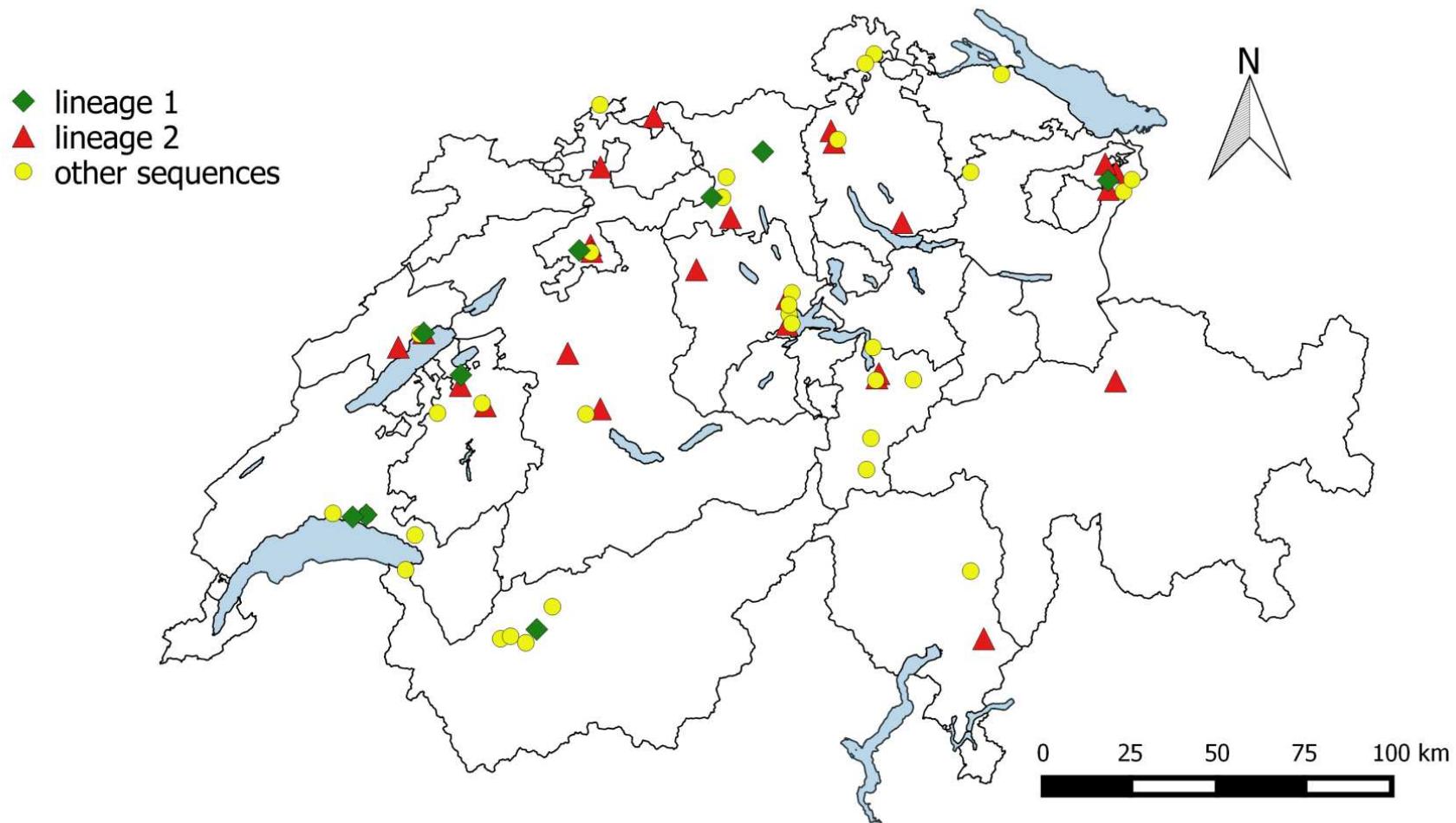


Fig. 4

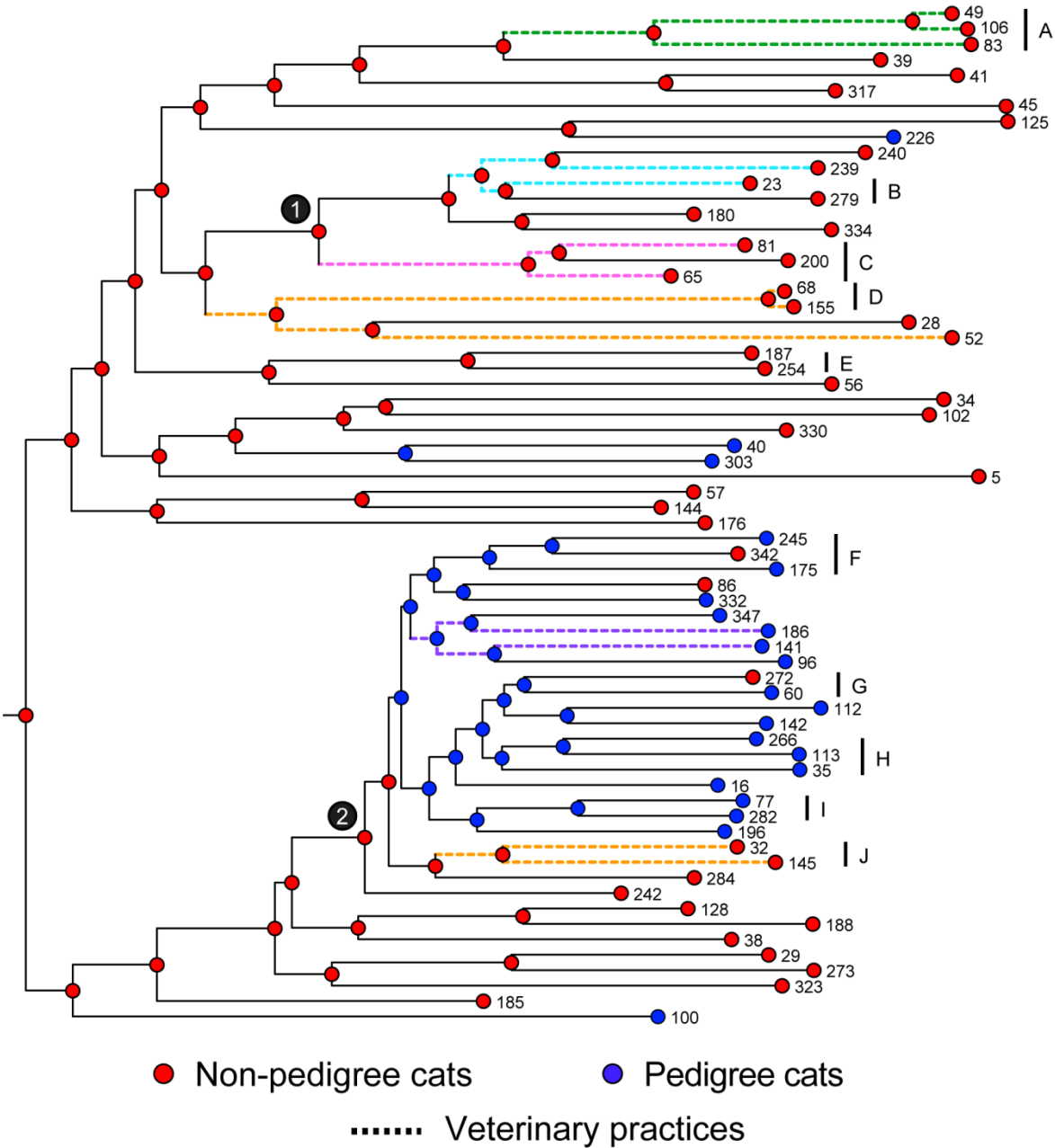
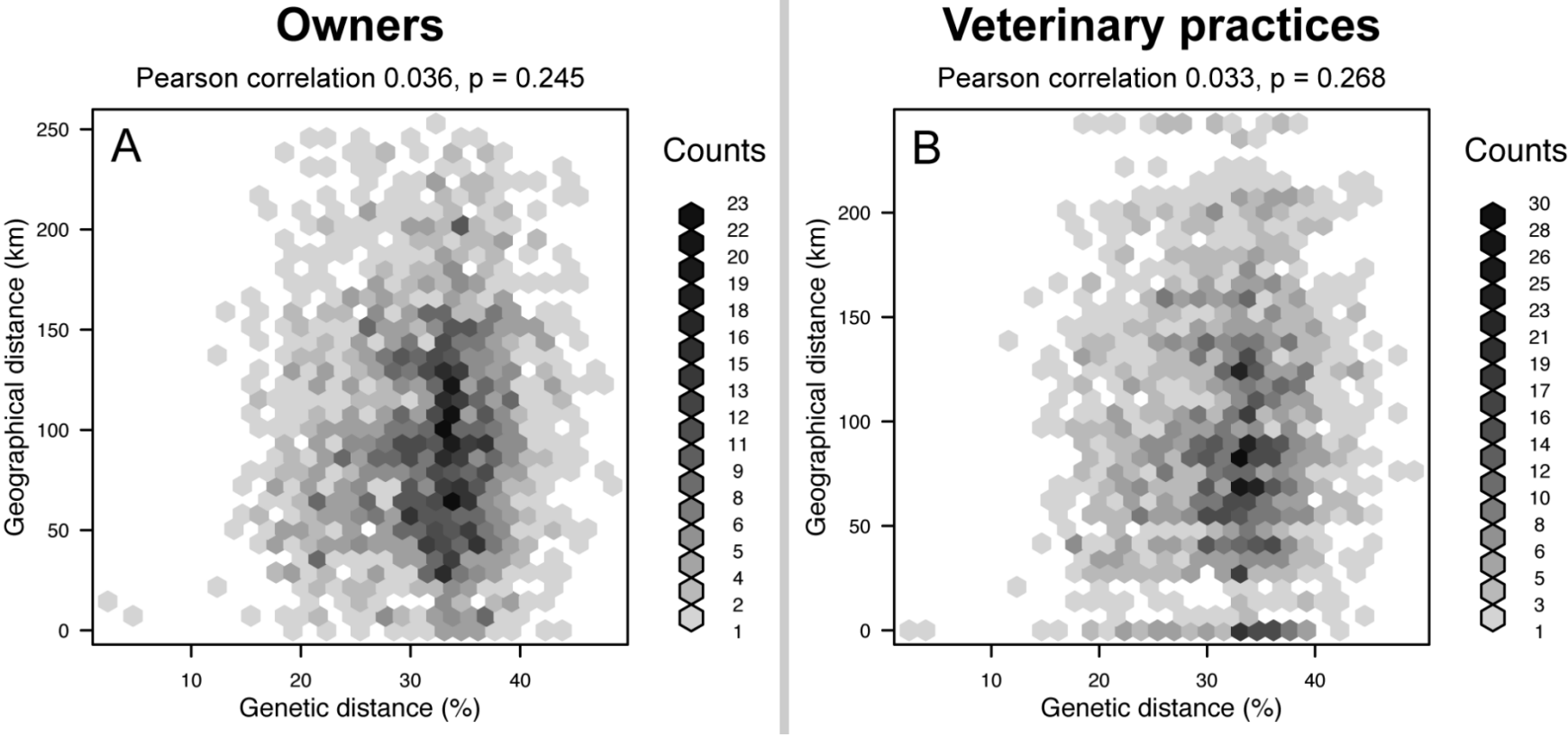


Fig. 5



Supplementary material**Table S1: Comparison of characteristics between the 45 non-pedigree and the 21 pedigree cats. P values < 0.05 are shown in bold.**

Parameter	Non-pedigree cats (n = 45)			Pedigree cats (n = 21)			P value
	Median	Lower 95% CI [#]	Upper 95% CI [#]	Median	Lower 95% CI [#]	Upper 95% CI [#]	
Age (years)	5.0	0.35	15.40	1.75	0.18	9.08	0.10
Parameter	Proportion	Lower 95% CI [#]	Upper 95% CI [#]	Proportion	Lower 95% CI [#]	Upper 95% CI [#]	P value
Sex (male)	0.67	0.51	0.80	0.52	0.30	0.74	0.27
Intact reproductive status	0.45	0.30	0.61	0.47	0.24	0.71	0.88
Multi-cat household*	0.70	0.54	0.83	0.95	0.76	1.00	0.02
Group housing*							0.16
2-3 cats/group	0.43	0.28	0.59	0.33	0.15	0.57	
4-5 cats/group	0.10	0.03	0.23	0.29	0.11	0.52	
>5 cats	0.17	0.07	0.31	0.33	0.15	0.57	
Husbandry type							<0.01
Private	0.82	0.68	0.92	0.71	0.48	0.89	
Cat breeder	0.00	0.00	0.00	0.29	0.11	0.52	
Other husbandry	0.18	0.08	0.32	0.00	0.00	0.00	
Outdoor access*	0.75	0.60	0.87	0.24	0.08	0.47	<0.01
Immunosuppressive therapy	0.22	0.11	0.37	0.00	0.00	0.00	0.02
Antibiotic therapy	0.40	0.26	0.56	0.14	0.03	0.36	0.04
Antiviral therapy*	0.02	0.00	0.12	0.05	0.00	0.25	0.55
FCV-vaccinated*	0.67	0.52	0.81	0.85	0.62	0.97	0.14
FCV vaccine strain*							0.01
Only F9	0.21	0.10	0.36	0.55	0.32	0.77	
Only G1/431	0.02	0.00	0.12	0.15	0.03	0.38	
Others	0.44	0.29	0.60	0.15	0.03	0.38	
Primary immunization* [‡]	0.49	0.32	0.66	0.79	0.54	0.94	0.03

[#]CI, confidence interval, * data missing for single cats (reproductive status, n = 5; multi-cat

household, n = 2; group housing, n = 3; outdoor access, n = 1; antiviral therapy, n = 1; FCV

vaccination, n = 3; FCV vaccine strain, n = 3; primary immunization, n = 10), [‡]primary

immunization was defined as two subsequent vaccinations within 2 to 6 weeks with the same

FCV vaccine strain

Fig. S1: Maximum likelihood phylogenetic tree estimated from 109 FCV capsid gene nucleotide sequences, including reference sequences and sequences generated in this study. Statistical support for the phylogenetic nodes was assessed using a bootstrap approach (100 replicates).

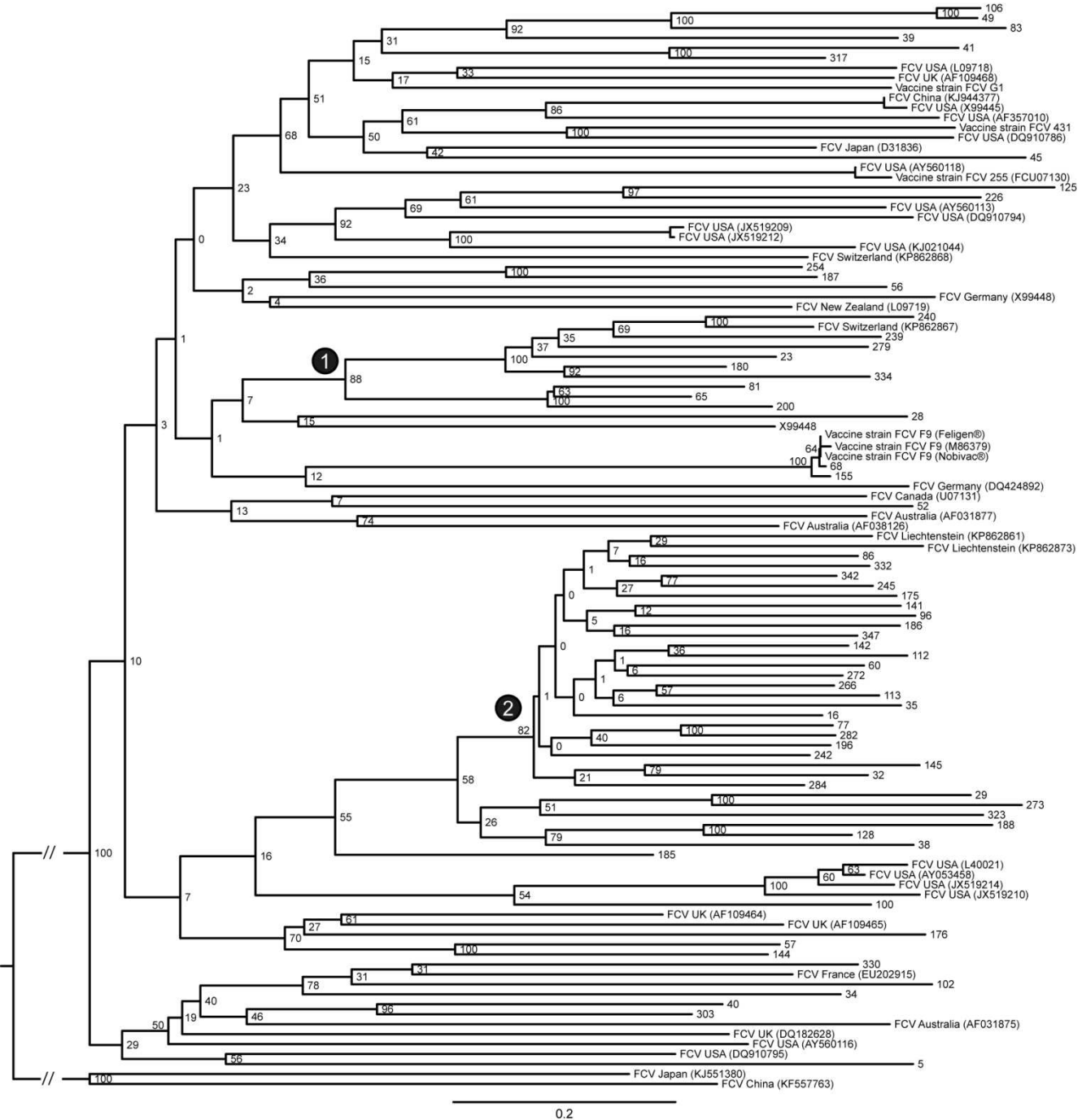


Fig. S2: Comparison of pairwise genetic distances (%) and pairwise geographical distances obtained from the samples in this study based on variable region E of VP1. Panels A and C show the correlations obtained if the pairwise geographic distances were calculated between the locations of the cat owners and included only samples isolated from non-pedigree cats. Panels B and D show the correlations obtained if the pairwise geographic distances were calculated between the locations of the veterinary practices and included only samples isolated from pedigree cats.

